Novel small molecule STAT3 inhibitor improves Teff:Treg balance of CD4 T cells from Multiple Sclerosis patients

Undergraduate Research Thesis

Presented in partial fulfillment for the requirements for graduation "with Honors Research Distinction in Neuroscience" in the undergraduate colleges of the Ohio State University

by

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April 2020

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Abstract

Multiple Sclerosis (MS) is a chronic autoimmune disorder of the central nervous system (CNS) in which the body's immune system attacks the fatty myelin sheath surrounding neurons, resulting in impaired neuronal signaling. Myelin-specific CD4 effector T cells (Teff) are pathogenic while CD4 T regulatory cells (Treg) are advantageous by suppressing Teff cells in both MS and its murine model, experimental autoimmune encephalomyelitis (EAE). Th17 cells are a major subset of Teff cells that mediate the formation of acute inflammatory CNS lesions and disease progression in EAE. Th17 cells are also implicated in MS pathogenesis. We focused on the Interleukin (IL)-6/STAT3 pathway in MS pathogenesis, as dysregulation of this pathway is observed in MS patients. IL-6 is a cytokine that signals through the transcription factor STAT3 to induce the development of Th17 cells, which produce a proinflammatory cytokine, IL-17. Moreover, IL-6 suppresses the generation of inducible Tregs (iTregs) and facilitates the Teff resistance to Treg-mediated suppression in MS patients, skewing the Teff:Treg balance towards Teff cells. This evidence suggests targeting STAT3 signaling may provide therapeutic benefit for MS. We developed a novel small molecule inhibitor, LLL12b, of STAT3. In previous studies, we found that LLL12b suppresses Th17 development and promotes iTreg development of murine and human CD4 T cells. In this study, we examined whether LLL12b could suppress the resistance of Teff cells from MS patients to Treg-mediated suppression. Using a CFSE-based suppression assay, our data show that LLL12b increases the Treg-mediated suppression on Teff cells, which may shift the functional Teff:Treg balance towards Tregs. Taken together, our novel small molecule STAT3 inhibitor may normalize the Teff:Treg balance of CD4 T cells and provide a novel therapeutic strategy for ameliorating disease progression in MS.

Introduction

The human immune system defends against infectious microbes as well as noninfectious foreign substances, products of damaged cells, and, under some circumstances, self-molecules. The immune system defends against microbes by using innate or adaptive immune responses. Innate immunity occurs within the first few hours or days after infection and utilizes mechanisms that are inherently present in the body, which allow for rapid responses. In contrast, adaptive immune responses occur several days after infection and increase in magnitude and defensive capabilities with each subsequent exposure to a particular microbe, or antigen[1].

The major class of cells involved in the adaptive immune response are lymphocytes, which consist of B cells and T cells. B cells are part of humoral immunity (one type of the adaptive immune response), and they block infections and eliminate extracellular microbes by secreting antibodies. Another type of adaptive immune response, called cell-mediated immunity, is mediated by T cells. There are mainly two types of T cells: helper T (Th) cells activate other immune cells to kill phagocytosed microbe and cytotoxic T lymphocytes (CTLs) directly destroy infected cells. B cells and T cells can be distinguished by the expression of cell surface proteins designated by a "CD" number. Most Th cells express CD4, whereas most CTLs express CD8. Lymphocytes also express specific antigen receptors, which allow the immune system to recognize and respond to millions of foreign antigens[1]. As myelin-specific CD4 T effector cells drive the formation of acute inflammatory demyelinating lesions and clinical relapses in the EAE model of MS, only CD4 T cells will be described further.

T cells originate from stem cells in the bone marrow and mature in the thymus. After maturation, T cells recirculate in the peripheral blood and secondary lymphoid organs, waiting until they come in contact with an antigen. When these circulating naïve T cells encounter an

antigen, a signaling cascade event occurs in which the T cells secrete cytokines, proliferate, and differentiate into effector cells, which are capable of eliminating the antigen[1,2]. Depending on the antigen, co-stimulators, and cytokines, naïve CD4 T cells can differentiate into distinct subsets of effector cells and produce different cytokines based on the transcription factor that is characteristically expressed (Figure 1). Th1 cells are produced in response to activation of the transcription factors STAT1, STAT4, and T-bet via interferon-γ (IFN-γ) and interleukin-12 (IL-12) signaling. Th1 cells are the major Teff subset involved in macrophage activation, which results in phagocytosis of microbes. Th2 cells develop when IL-4 activates STAT6, and they mainly function to promote eosinophil- and mast cell-mediated reactions to eliminate infection and promote tissue repair by producing IL-4, IL-5, and IL-13. A third subset of CD4 Teff cells are Th17 cells, which produce IL-17 in response to IL-6, IL-1, and IL-23[1]. The development of Th17 cells is dependent on STAT3 activation, as endogenous Th17 cells are absent in $STAT3^{-/-}$ mice[3]. Th17 cells mainly recruit neutrophils to sites of infection, producing inflammation to eliminate bacterial and fungal microbes[1]. Previous studies have shown that Th1 and Th17 subsets of CD4 T effector cells are encephalitogenic in EAE and are implicated in MS pathogenesis[4,5].

Self-tolerance refers to the ability of the immune system to recognize self-antigens but not respond to them, and this property is essential for the functioning of a normal immune system. Most thymic-derived CD4 T cells with high affinity for self-antigens are targeted for deletion, but some of these T cells differentiate into Tregs that inhibit Teff responses to self-antigens. CD4 Tregs express high levels of CD25, the IL-2 receptor α chain, and a transcription factor called forkhead box protein 3 (Foxp3)[1]. In experimental settings, naïve T cells in the periphery can also acquire Foxp3 expression. For instance, in the presence of TGF-β, retinoic acid (RA), and IL-2, naïve T cells are induced to differentiate into $FoxP3^{+}$ iTregs in-vitro[6,7,8,9].

iTregs are typically present at mucosal surfaces and are essential for oral tolerance, although they have also been observed in the spinal cords of mice with experimental autoimmune encephalomyelitis[10]. The balance between Teff:Treg cells is critical for the normal function of the immune system, since a Teff:Treg balance skewed towards Teffs favors autoimmunity.

MS is the most common cause of neurologic disability in young adults after trauma, with recent reports estimating a prevalence of nearly 750,000 cases in the United States alone[11]. Age of onset ranges from 20-40 years old, and women are affected 2-3 times more than men[11,12]. MS is also more frequent in regions at a higher latitude, though this gradient has been decreasing over the last five decades[11,13]. MS is a heterogeneous disease and consists of three main subtypes: relapsing-remitting MS (RRMS), primary progressive MS (PPMS), and secondary progressive MS (SPMS). RRMS is characterized by periods of attacks of neurologic symptoms followed by periods of partial or full recovery, and approximately 85% of people with MS are initially diagnosed with RRMS. SPMS follows an initial relapsing-remitting course and transitions into progressive worsening of symptoms, whereas PPMS is characterized by an accumulation of neurologic disability from the onset of symptoms, without any remissions. The formation of gadolinium-enhancing lesions in the brain and spinal cord correlate with symptoms, including fatigue, numbness or tingling, vision problems, and weakness, among other things[14].

Initiation and progression of MS occurs in three stages: inflammation, demyelination, and axonal damage. The disease process is mediated by autoreactive CD4 T cells that target myelin proteins. Normally, the blood brain barrier (BBB) blocks entry from T cells in the periphery, but cell surface molecules on activated T cells allow migration across the BBB. Once in the CNS, the T cells must be reactivated to mediate pathology. Antigen-presenting cells (APCs), including dendritic cells, macrophages, microglia, or B cells, process proteins into peptide fragments and

present them to T cells, mainly through major histocompatibility complex class II. Finally, T cells are reactivated and produce cytokines, such as IFN-γ or IL-17, which in turn activate microglia and macrophages. The recruitment of T cells, microglia, and macrophages results in the formation of lesions in the gray and white matter. Microglia and macrophages produce other inflammatory mediators that, in addition to the cytokines, can damage myelin. Without any myelin, axons remain exposed and are subject to transection, leading to neuronal death[12,15].

There are currently 15 FDA-approved disease-modifying therapies (DMTs) available to MS patients, including injectables, oral drugs, and monoclonal antibodies. Injectable drugs, like interferon beta and glatiramer acetate, are safer than other DMTs available but are not as efficacious. Oral drugs, such as dimethyl fumarate and fingolimod, and monoclonal antibodies, such as natalizumab and alemtuzumab, have higher efficacy, but they can cause serious adverse events, including progressive multifocal leukoencephalopathy (PML), a potentially fatal, viral infection of the brain[16,17]. In addition to side effects, patients often experience unpleasant reactions after injections and infusions, highlighting the need for novel oral therapies.

Dysregulation of the IL-6/STAT3 signaling pathway plays an important role in the pathogenesis of MS and other autoimmune diseases. IL-6 is elevated in plasma[18] while IL-6 mRNA and protein levels are elevated in the CNS of MS patients[19,20]. In addition, B cells from MS patients secrete significantly more IL-6 than healthy controls (HC)[21]. CD4 T cells from MS patients have significantly more IL-6 receptors (IL-6R) than HC[22] and the expression of phosphorylated STAT3 (pSTAT3) in peripheral blood mononuclear cells (PBMC) from RRMS patients strongly correlates with MS disease activity[23]. These pieces of evidence suggest targeting STAT3 may normalize dysregulated IL-6/STAT3 signaling and provide therapeutic benefits for MS patients.

IL-6/STAT3 signaling is a key regulator of Teff:Treg balance of CD4 T cells. IL-6 binds to the IL-6 receptor alpha (IL6-Rα) and GP130 on naïve CD4 T cells to form an IL-6/IL-6Rα/GP130 heterodimer[2,24,25]. Two heterodimers form an active heterohexameric signaling complex, leading to downstream STAT3 activation and Th17 development[3]. In turn, Th17 cells produce the pro-inflammatory cytokine IL-17, which is pathogenic in MS and EAE[5,26]. Additionally, activation of the IL-6/STAT3 signaling pathway blocks the development of iTregs. For instance, IL-6/STAT3 signaling in murine CD4 T cells inhibits the induction of $FoxP3^-$ into $FoxP3^+$ Tregs in-vivo[27]. As a result, dysregulated IL-6/STAT3 signaling skews Teff:Treg balance toward an enhanced Teff response, favoring the development of autoimmunity. In-vitro derived iTregs are functionally suppressive in EAE[28] and can be used to augment and restore regulatory networks in situations where nTregs are exhausted or defective[29]. iTregs can also be generated in large numbers and regulate inflammation by direct suppression of endothelial activation and leukocyte recruitment[30], making them an important tool in the treatment of autoimmune diseases. Recent studies have shown that Teff cells are resistant to Treg-mediated suppression in MS and other autoimmune diseases. In particular, Teff cell resistance involves IL-6/STAT3 signaling. For example, Schneider et al. (2013) observed increased IL-6Rα expression and elevated IL-6 signaling as measured by pSTAT3 in RRMS subjects, and blockade of pSTAT3 restored functional suppression of Teff cells by Tregs[31,32].

We have developed LLL12b, a novel small molecule inhibitor of STAT3 that binds to the SH2 domain of STAT3, preventing STAT3 phosphorylation and dimerization (Figure 3). As a small molecule, LLL12b has the potential to penetrate the BBB and may offer enhanced suppression of encephalitogenic T cells in the CNS. We have previously shown that LLL12b reduces IL-17 production and promotes iTreg development in mice with EAE and in human

peripheral blood mononuclear cells (PBMCs). Thus, we hypothesized that by inhibiting STAT3, LLL12b could restore iTreg-mediated suppression on Teff cells and normalize the balance of Teff:Treg of CD4 T cells from MS patients. To test our hypothesis, we performed carboxyfluorescein succinimidyl ester- (CFSE) based suppression assays. CFSE is a cell stain used to track multiple generations of cell division and proliferation (Figure 4). We determined if LLL12b improves iTreg-mediated suppression of murine myelin-specific CD4 T cells as well as PBMCs from treatment-naïve MS patients.

Methods

Generation of murine iTregs:

Splenocytes were isolated from three B6 mice and incubated for 72 hours with 500 u/mL recombinant mouse IL-2 (rmIL-2), 5 ng/mL rhTGF-β, and 10 nM RA on a 24-well plate coated with anti-mouse CD3/CD28 antibodies. After 72 hours, the cells were collected in a 50 mL conical tube, and the plate was washed with warm PBS to collect any remaining cells. Some cells were used for flow cytometry, and the rest of the cells were used for the suppression assay.

Labelling myelin-specific CD4 T cells with CFSE:

Splenocytes from three 2D2 mice that are specific for MOG 35-55 were isolated and resuspended in a 50 mL conical tube with PBS at a concentration of $20x10^6$/mL. CFSE was diluted in PBS to a final concentration of 100 uM. The diluted CFSE (100 uM) was added to 2D2 splenocytes that made the final concentration of CFSE 5 uM. The CFSE-cell suspension was then incubated in a 37^{o}C water bath for 10-15 minutes. PBS was added at 9x the original volume to the cells, and the cells were spun down at 1300 rpm for 8 minutes at 23^{o}C. Cells were

washed again with 10 mL of PBS and spun down using the same settings mentioned previously. The cells were resuspended to a final volume of $2x10^6$/mL.

Murine suppression assay:

The CFSE-labelled cells from 2D2 mice were mixed with 10 ug/mL MOG 35-55, and 25 ng/mL rmIL-6. The iTregs were collected, counted, and resuspended in EAE media at a concentration of $2x10^6$/mL. The CFSE-2D2 cells and iTregs were aliquoted to a 24-well plate in the following Teff:Treg ratios: 1:0, 1:1, 2:1, and 4:1. DMSO was added to the control wells for vehicle control. The diluted LLL12b was added to half of the experimental wells for a final concentration of 0.25 uM and the other half of the experimental wells for a final concentration of 0.5 uM. The cells were incubated for 4 days. % suppression was determined as 100-(% proliferating $CFSE^+$ cells in each ration/% proliferating $CFSE^+$ cells without Tregs)100.

Recovery of frozen PBMCs:

Peripheral blood mononuclear cells ($1x10^8$) from three treatment-naïve MS patients were taken out of liquid nitrogen and put in a 37^{o}C water bath immediately. Cells were transferred into cold PBS after thawing and spun down at 1300 rpm for 8 minutes at 4^{o}C. The cells were washed again with cold PBS and spun down at 1300 rpm for 8 minutes at 4^{o}C. Next, cells were resuspended in 20 mL of human medium and transferred to a 75 mm flask/patient. Cells incubated for 2 hours at 37^{o}C. After the incubation period, the cells were passed through a strainer and spun down at 1300 rpm for 8 minutes at 23^{o}C. Cells were resuspended in 2 mL of human medium and counted.

Generation of human iTregs:

15 ng/mL rhIL-2 and 10 nM RA were added to a mix containing isolated human PBMCs, human media, and 5 ng/mL rhTGF-β1. The cell mix was pipetted onto a 48-well plate (1 mL per well) and incubated for 72 hours at 37^{o}C.

Labelling PBMCs with CFSE. CFSE was diluted in PBS to a final concentration of 100 uM. The diluted CFSE (100 uM) was added to PBMCs that made the final concentration of CFSE at 1 uM. The CFSE-cell suspension was incubated in a 37^{o}C water bath for 15 minutes. PBS was added at 9x the original volume to the cells, and the cells were spun down at 1300 rpm for 8 minutes at 23^{o}C. Cells were washed again with 10 mL of PBS and spun down using the same settings mentioned previously. Cells were resuspended in human medium.

Pre-treatment of Teff cells with LLL12b. LLL12b was diluted to a concentration of 0.25 uM, and DMSO was used as a control. CFSE-labelled Teff cells were resuspended in human medium and incubated for one hour at 37^{o}C in a 24-well plate with rhIL-6 and LLL12b or DMSO. After incubation, cells were collected into a 15 mL conical tube, and the plate was washed with 1 mL of human medium and collected into each tube. The cells were spun down at 1300 rpm for 8 minutes at 23^{o}C and resuspended in human medium.

Human suppression assay:

iTregs were collected and counted and aliquoted with the DMSO- or LLL12b-treated CFSE cell mix onto an anti-human (αh) CD3/CD28 coated 48 well plate (1 mL/well) according to the following ratios of Teff:Treg: 4:1, 8:1, and 16:1. There was also a condition containing only CFSE-labelled Teff cells, which was used to monitor the natural proliferation of Teff cells. The cells were incubated for 5 days at 37^{o}C. % suppression was determined as 100-(% proliferating $CFSE^{+}$ cells in each ration/% proliferating $CFSE^{+}$ cells without Tregs)100.

Flow cytometry

Surface Staining:

Murine cells or PBMCs from culture were microcentrifuged at 2000 rpm for 1 minute at 23^{o}C. The supernatant was vacuumed out, and the pellets were resuspended in 50-100 uL of staining buffer (1% BSA in PBS). The cells were then stained with surface antibodies and Fc-block for 30 minutes at 4^{o}C in the dark and washed three times with staining buffer. Cells were resuspended in 200 uL of staining buffer and transferred to FACS tubes. 80,000-100,000 live cell events were acquired on a FACSCantoII and analyzed using FlowJo software.

Intracellular staining for Foxp3:

Murine cells or PBMCs were surface stained as described previously. After the staining period, the cells were washed three times with staining buffer and then fixed and permeabilized using a 1x fix/perm buffer (eBioscience) for 60 minutes on ice in the dark. The cells were then washed twice with staining buffer and placed in a 4^{o}C fridge overnight. The following day, 1x perm/wash buffer was added (eBioscience), and the cells were placed on ice for 20 minutes in the dark. Next, the cells were washed one time with 1x perm/wash buffer and then intracellularly stained with an antibody mix consisting of Foxp3 and Fc-block for 30 minutes on ice in the dark. After incubating, the cells were washed three times with 1x perm/wash buffer. After the last wash, the cells were resuspended in 200 uL of 1x perm/wash buffer and transferred to FACS tubes. 80,000-100,000 live cell events were acquired on a FACSCantoII and analyzed using FlowJo software.

Phospho staining:

Human PBMCs were recovered as described previously and aliquoted to a 24 well plate (1 mL/well) plus rhIL-6, and DMSO or 0.25 uM LLL12b and incubated for 30 minutes at 37^{o}C.

1 mL of pre-warmed Fix/Perm Buffer (BD biosciences) was added to each well immediately after incubation. The cells were mixed, transferred to 15 mL conical tubes, and placed in a 37ºC water bath for 10-12 minutes. Next, the cells were spun down at 600 G for 8 minutes at 23ºC. The supernatant was vacuumed out, and the cells were vortexed to disrupt the cell pellet. 1 mL of chilled Perm Buffer III was slowly added to the cells to permeabilize them. The cells were vortexed and placed on ice for 30 minutes. 5 mL of stain buffer (0.5% FBS/PBS) was added to each tube and spun down at 600 G for 8 minutes at 4ºC. The supernatant was vacuumed out, and 400 uL of stain buffer was added to each tube. The cells were transferred to a flow plate, spun down at 2000 rpm for 2 minutes at 4ºC, and washed three times with stain buffer. The cells were stained with either isotype control or pSTAT3 antibodies plus Fc-block for one hour at room temperature in the dark. The cells were washed three times, resuspended in 200 uL of stain buffer, and transferred to FACS tubes. 80,000-100,000 live cell events were acquired on a FACSCantoII and analyzed using FlowJo software.

Results

LLL12b enhances iTreg-mediated suppression of murine Th17 cells

We first examined whether LLL12b may enhance Treg-mediated suppression of murine myelin-specific Th17 cells using CFSE-based suppression assays. Splenocytes from three WT/B6 mice were activated with MOG 35-55 plus TGF-β, rmIL-2, and RA for 3 days to generate iTregs. The development of iTregs was determined by intracellular staining of Foxp3 (Figure 4). The cells were collected and stained with viability dye and CD4 and CD25 antibodies, followed by intracellular staining with Foxp3. Live CD4+ cells were gated on CD25 (a marker of activated Tregs) and Foxp3 (an essential transcription factor expressed by Tregs) to

quantify iTregs. Meanwhile, splenocytes from three 2D2 mice that are specific for MOG 35-55 were isolated and labeled with CFSE. We then co-cultured the iTregs with CFSE-labelled splenocytes from 2D2 mice at Teff:Treg ratios of 1:0, 4:1, 2:1, and 1:1 and activated them with MOG 35-55 and rmIL-6 for 4 days in the presence of DMSO, 0.25 uM LLL12b, or 0.5 uM LLL12b (Figure 6). The proliferation of MOG 35-55 specific CD4 T cells was determined by flow cytometric analysis of CFSE on $CD4^+$ T cells (Figure 6a). The $CFSE^-$ population represents proliferating MOG 35-55 specific CD4 T cells while the $CFSE^+$ population is not proliferating. Our data show that the proliferating $CFSE^-$ cells decrease (36%, 24%, and 21%) when there are more iTregs in the culture (Teff:Treg ratio: 4:1, 2:1, and 1:1) (Figure 6a, 6b). The highest amount of proliferation occurred when no Tregs were present (Teff:Treg ratio at 1:0), and the lowest amount of proliferation occurred at 2:1 and 1:1 ($p<0.001$), suggesting the iTregs we generated are functionally active by suppressing the proliferation of MOG 35-55-specific CD4 T cells. Furthermore, % proliferating cells were notably lower in LLL12b treated groups compared to vehicle control DMSO group (Figure 6c), suggesting LLL12b treatment leads to decreased proliferation. % suppression was determined as 100-(% proliferating $CFSE^+$ cells in each ratio/% proliferating $CFSE^+$ cells without Tregs)100. LLL12b significantly reduced Teff resistance to Treg-mediated suppression, as shown by % inhibition (Figure 6d). This data suggests that by inhibiting STAT3 in murine myelin-specific CD4 T cells, Treg-mediated suppression on Teff cells was improved.

LLL12b inhibits the phosphorylation of STAT3 in CD4+ T cells from MS patients

To determine if LLL12b may suppress the resistance of human PBMCs from MS patients to Treg-mediated suppression, we first determined if LLL12b inhibits pSTAT3 in human CD4 T

cells from MS patients. PBMCs from 6 treatment-naïve MS patients were activated with plate bound αhCD3/CD28 plus rhIL-6 for 30 minutes, in the presence of 0.25 uM of LLL12b or DMSO. DMSO- and LLL12b-treated cells were stained with a CD4 surface antibody and either an isotype control antibody or a pSTAT3 antibody for one hour. To determine levels of pSTAT3 in MS patients, we ran flow cytometry and focused on $CD4^+pSTAT3^+$ cells. $pSTAT3^+$ cells represent $CD4^+$ T cells in which the IL-6/STAT3 pathway is activated. Compared to our vehicle control, LLL12b significantly reduced levels of pSTAT3 ($p<0.05$), meaning LLL12b suppresses the phosphorylation of STAT3 in human CD4+ T cells from MS patients (Figures 7a). % $pSTAT3^+$ cells in the DMSO and LLL12b-treated conditions was determined and compared for each patient. All MS patients showed a reduction in % $pSTAT3^+$ CD4 T cells, with some patients exhibiting greater reductions than other patients (Figure 7b), indicating that LLL12b may be variably effective in suppressing STAT3 phosphorylation in human CD4 T cells from MS patients. This data suggests LLL12b suppresses activation of the IL-6/STAT3 signaling pathway.

LLL12b reduces the resistance of CD4 T cells from MS patients to iTreg-mediated suppression

We examined whether LLL12b could restore Treg-mediated suppression on Teff cells by conducting CFSE-based suppression assays. Human PBMCs from 3 treatment-naïve MS patients were activated with plate bound αhCD3/CD28 plus rhTGF-β1, rhIL-2, and RA for 3 days to generate iTregs. The development of iTregs was determined by intracellular staining of Foxp3, as described previously. PBMCs from the same patients were labeled with CFSE and cultured with 0.25 uM of LLL12b or DMSO for 1-2 hours. The LLL12b-treated CD4 T cells were then mixed with iTregs generated from the same patients at Teff:Treg ratios of 1:0, 4:1, 8:1, and 16:1.

The proliferation of human Teff cells was determined by flow cytometric analysis of CFSE on $CD4^+$ T cells. % suppression was determined as 100-(% proliferating $CFSE^+$ cells in each ratio/% proliferating $CFSE^+$ cells without Tregs)100. We found that Tregs suppress the proliferation of human CD4 T cells in a dose-dependent manner (Figure 8a). % non-proliferating $CFSE^+$ cells increased (54%, 70%, and 76%) in the DMSO condition when there were more iTregs in the culture (Teff:Treg ratio 16:1, 8:1, 4:1), suggesting the human Tregs we generated are functionally active. LLL12b enhanced Treg-mediated suppression of Teff cells compared to DMSO, with optimal suppression occurring at a Teff:Treg ratio of 4:1, followed by 8:1 and 16:1. Suppression varied among patients, but all patients showed increased Treg-mediated suppression of Teff cells when given LLL12b compared to DMSO, even in the 16:1 condition (Figure 8c). This data suggests that Treg-mediated suppression on CD4 Teff cells from MS patients can be improved by inhibiting STAT3.

Discussion

MS patients have a skewed Teff:Treg balance compared to healthy individuals, resulting in excessive Teff responses. The IL-6/STAT3 signaling pathway is a crucial regulator of the Teff:Treg balance, and aberrant signaling results in the production of highly encephalitogenic Th17 cells, blockage of iTreg development, and resistance of CD4 Teff cells to Treg-mediated suppression. We hypothesized that LLL12b, a novel small molecule STAT3 inhibitor, could enhance Treg suppressive function on Teff cells in CD4 T cells from MS patients. Our data supported our hypothesis in that LLL12b reduced resistance of CD4 Teff cells to Treg-mediated suppression. In murine CFSE-based suppression assays, we established that LLL12b promoted increased suppression of Teff cells when the Teff:Treg ratio was 2:1 and 4:1. LLL12b also

reduced Teff resistance to Treg-mediated suppression in PBMCs from treatment-naïve MS patients. We observed enhanced suppression of CD4 Teff cells by Tregs in a dose-dependent manner. The optimal ratio for suppressing CD4 Teff cells occurred at a Teff:Treg ratio of 4:1, followed by 8:1 and 16:1. We also found that LLL12b significantly reduced pSTAT3 levels, indicating that we effectively targeted STAT3 rather than a downstream component of the IL-6/STAT3 signaling pathway. Taken together, our data suggest that LLL12b has the potential to normalize the Teff:Treg balance in CD4 T cells and is a promising lead compound for the treatment of MS.

In the murine suppression assays, we added LLL12b into the culture containing CFSE-labelled 2D2 cells and iTregs, meaning both Treg and Teff cell populations were exposed to LLL12b treatment. Although our previous experiments show LLL12b promotes Treg development in mice and human PBMCs, there is no TGF-β added to induce iTreg development during the suppression assays. Therefore, the possibility that LLL12b exerts its function through promoting iTreg development during the suppression assays is minimal. To rule out this possibility, we pretreated PBMCs with LLL12b, followed by co-culture of LLL12b-treated CD4 T cells with iTregs for human suppression assays. In this way, we could be certain that LLL12b was directly exerting its effects on the Teff cell population.

It is important to note that the viability of frozen PBMC samples from MS patients presented a limitation to our study. Murine cells are relatively stable in culture, so we faced little difficulty in establishing the efficacy of LLL12b in murine cell culture. To test LLL12b in humans, we used cryopreserved PBMCs from MS patients. Unfortunately, cell viability after thaw is highly variable among patients; thus, we only utilized samples that were easily recoverable and generated enough cells to use through the entirety of the experiment. To increase

our external validity, we would like to run the same experiments with fresh PBMCs from MS patients, as the cell composition of frozen PBMCs is relatively similar to fresh PBMCs, so as to eliminate the detrimental recovery process. Even with this limitation, our data highlight the potential of targeting the IL-6/STAT3 signaling pathway to ameliorate CNS autoimmunity.

Most current DMTs for the treatment of RRMS come with a host of drawbacks. Injectable DMTs, such as IFN-β and glatiramer acetate, are safe and have been the first-line treatment options for two decades. However, they are only moderately effective and are poorly tolerated due to injection-related adverse events[12]. More recent DMTs have both higher efficacy and increased risk of serious adverse events like PML. Furthermore, monoclonal antibodies may not penetrate the CNS as effectively, limiting the suppression of CNS-infiltrating Teff cells and the promotion of Treg development in the CNS. Small molecules have the advantage of penetrating the BBB to directly exert their function, and an oral drug like LLL12b could eliminate the complications of injection or infusion-related reactions[33].

Tocilizumab, a humanized αIL-6R monoclonal antibody, has proven efficacious for the treatment of rheumatoid arthritis[34], giant cell arteritis[35], and juvenile idiopathic arthritis[36]. Recently, our lab showed that three novel small molecule IL-6 inhibitors, madindoline-5 (MDL-5), MDL-16, and MDL-101 suppress IL-17 production in myelin-specific CD4 T cells and promote iTreg development in-vitro[33]. confirming that targeting IL-6/STAT3 signaling may provide therapeutic benefits for MS.

Although loss of STAT3 in CD4 T cells prevents development of CNS inflammatory diseases, STAT3 is implicated in numerous other functions, including induction of an acute phase response in hepatoma cells, stimulation of proliferation of B cells, activation of terminal differentiation and growth arrest in monocytes, and maintenance of the pluripotency of

embryonic stem cells[25]. Furthermore, IL-10 requires STAT3 activation for its anti-inflammatory properties on macrophages[37]. Given that MS patients have unusually elevated IL-6/STAT3 signaling, normalizing dysregulated STAT3 activation may provide better therapeutic effects with potentially fewer side effects. Because of the complex nature of STAT3, future studies could look at improving LLL12b's bioavailability and drug delivery methods to more efficiently target CD4 T cells.

List of Figures

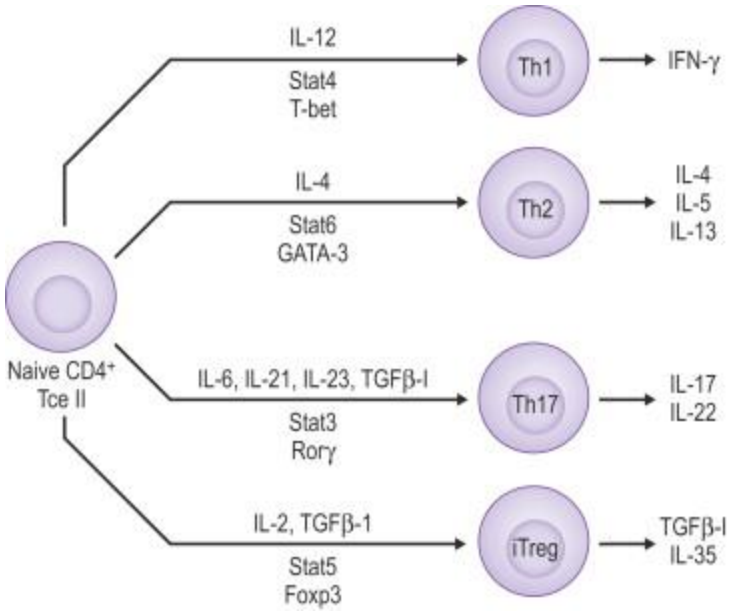

Figure 1. T cell differentiation. Naïve $CD4^+$ T cells differentiate into Teff cells or Tregs depending on costimulatory factors[38].

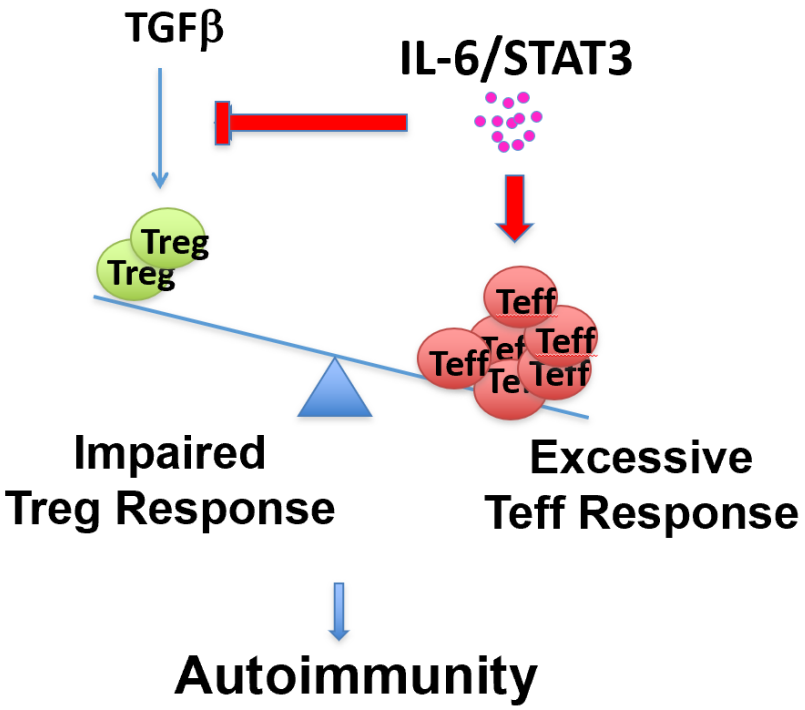

Figure 2. IL-6/STAT3 signaling regulates Th17:Treg balance. IL-6/STAT signaling inhibits Treg development and promotes excessive Teff cell responses. A skewed Teff:Treg balance toward Teff responses results in autoimmunity.

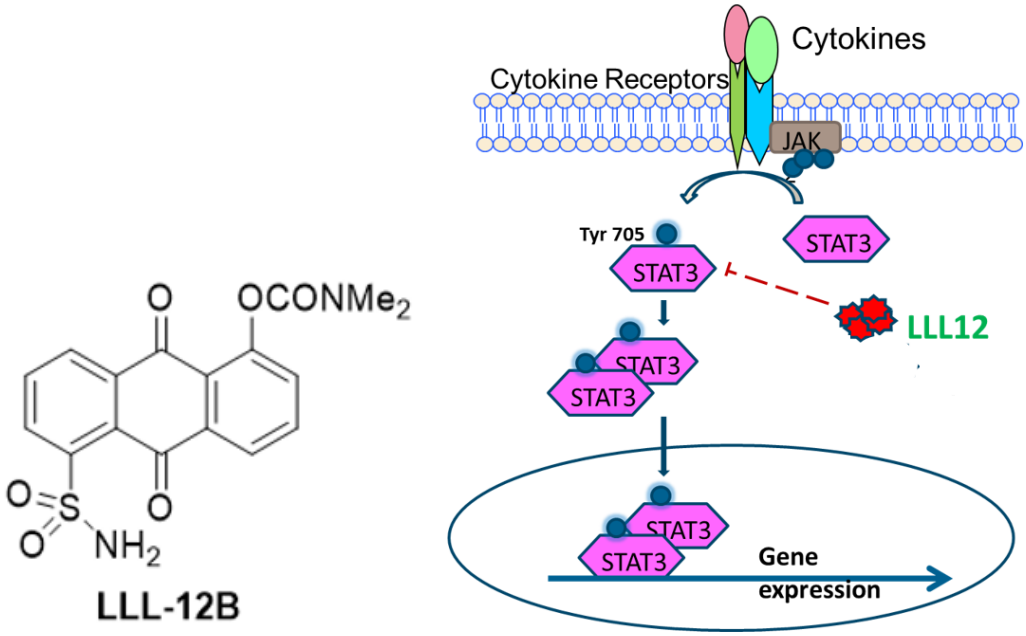

Figure 3. Molecular structure and mechanism of action of LLL12b. LLL12b is a prodrug of LLL12 and binds to the SH2 domain of STAT3, preventing STAT3 phosphorylation and dimerization.

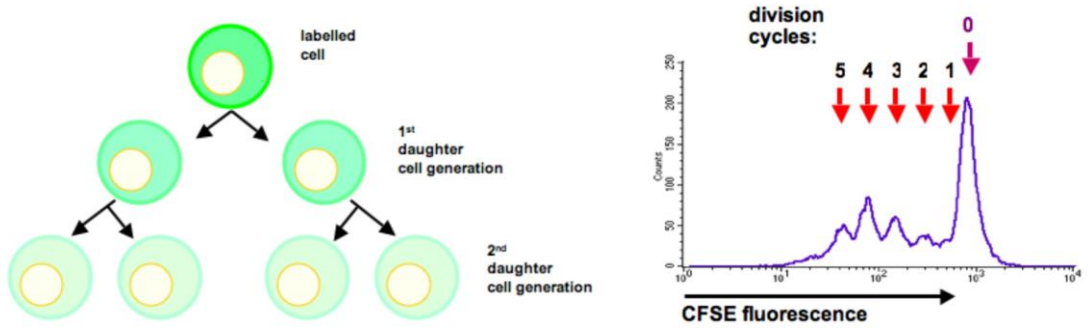

Figure 4. CFSE proliferation. (A) With each cell division, CFSE-labelled cells become progressively less fluorescent. (B) Dilution of the CFSE dye can be tracked by flow cytometry as cells proliferate over time. Numbers above the arrows represent each major cell division in CFSE-labelled human $CD4^+$ T cells after 4 days of in-vitro stimulation[39].

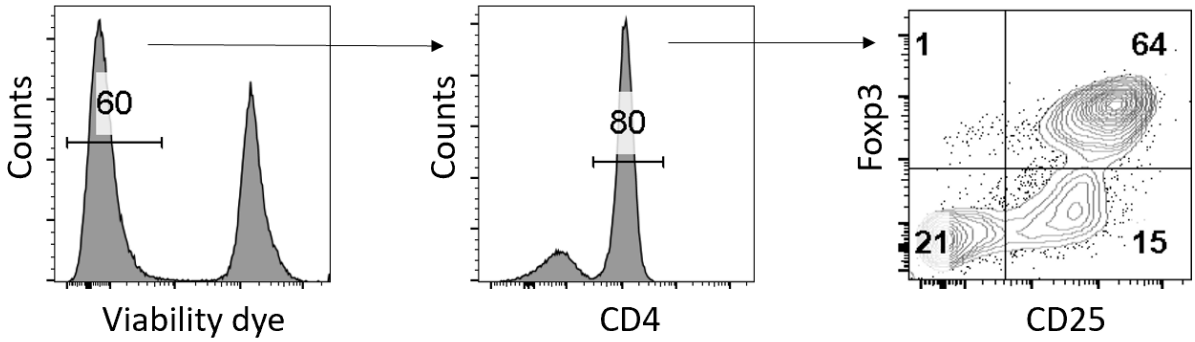

Figure 5. Development of murine iTregs. First, we stained with viability dye to distinguish live and dead cells. The viable cells were gated on CD4, and the $CD4^+$ cells were gated on CD25 (a marker of activated Tregs) and Foxp3 (an essential transcription factor expressed by Tregs). iTregs were defined as $CD4^+CD25^+Foxp3^+$.

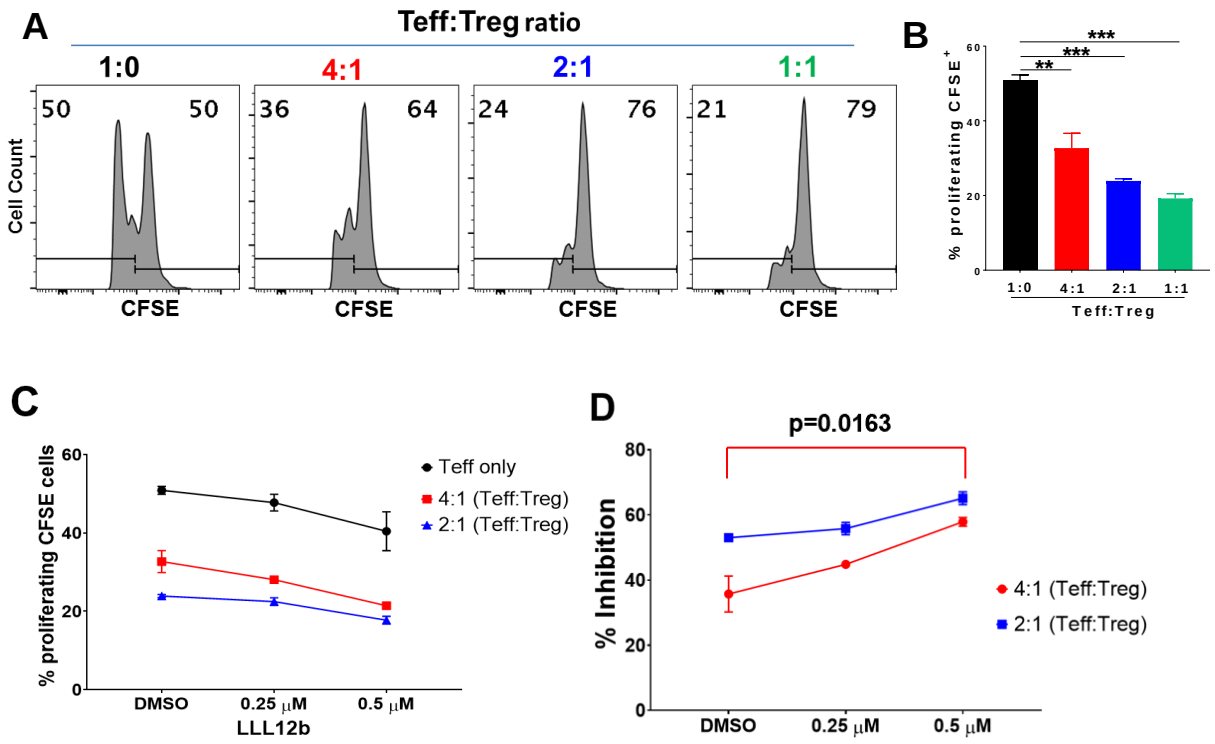

Figure 6: LLL12b enhances Treg-mediated suppression of myelin-specific CD4 T cells.

Splenocytes from three B6 mice were activated with MOG 35-55 plus TGF-β, IL-2, and RA for 3 days. The cells were then co-cultured with CFSE-labelled splenocytes from three 2D2 mice that are specific for MOG 35-55 at various ratios and activated with MOG 35-55 and rmIL-6 for 4 days in the presence of DMSO, 0.25 uM, or 0.5 uM LLL12b. (A) Representative flow plot of DMSO-treated CD4 T cells. CFSE was determined by flow cytometry. (B) % proliferating $CFSE^+$ cells were compared with one-way ANOVA (n=3) from (A). (C) % proliferating $CFSE^+$ cells in DMSO- or LLL12b-treated groups at three Teff:Treg ratios from (A) was calculated. Represents data from one experiment (D) Combined % inhibition of $CFSE^+$ cells in DMSO- or LLL12b-treated groups were compared with two-way ANOVA (n=3) at two Teff:Treg ratios. All error bars denote s.e.m. **p<0.01; ***p<0.001.

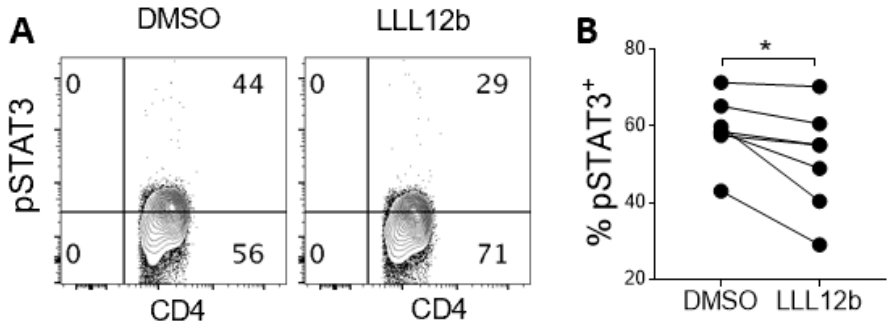

Figure 7. LLL12b inhibits the phosphorylation of STAT3 in CD4 T cells from MS patients. PBMCs from 6 treatment-naïve MS patients were activated with plate bound αhCD3/CD28 plus rhIL-6 for 30 minutes in the presence of 0.25 uM of LLL12b or DMSO. pSTAT3 was determined by flow cytometry. Cells were gated on $CD4^+$ T cells. (A) Representative flow plot of pSTAT3 in LLL12b or DMSO-treated group from one MS patient. (B) pSTAT3 in LLL12b-treated group and DMSO-treated group from 6 treatment-naïve MS patients were summarized and compared with Wilcoxon matched-pairs signed rank test for significance. *denotes $p<0.05$.

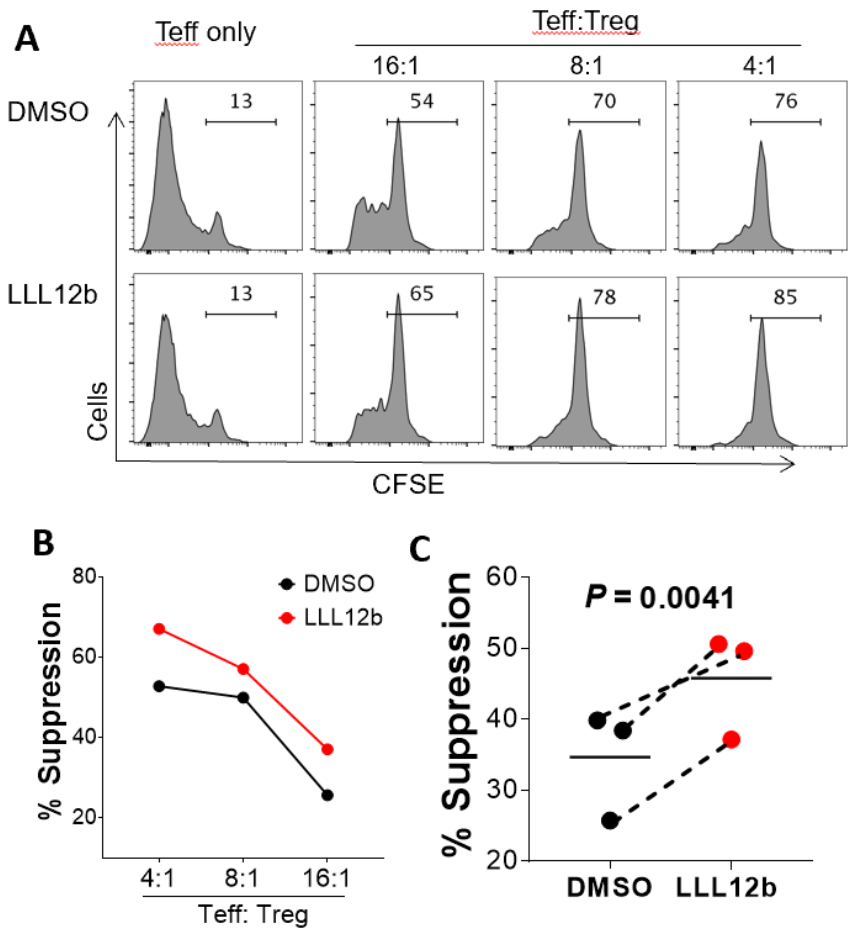

Figure 8. LLL12b reduces the resistance of CD4 T cells from MS patients to Treg-mediated suppression. PBMCs from three treatment-naïve MS patients were activated with αhCD3/CD28 plus rhTGFβ, rhIL-2 and RA for 3 days to generate Tregs. PBMCs from the same patients were labeled with CFSE and cultured with 0.25μM of LLL12b or DMSO for 1-2hrs. The treated CD4 T cells were mixed with iTregs generated from the same patient at four different ratios and cultured for 5 days. (A) Flow cytometric analysis of proliferation of CFSE-stained $CD4^+$ T cells from one treatment-naïve MS patient. (B) % suppression by Tregs in DMSO- or LLL12b-treated group at three Teff:Treg ratios from (A) was calculated. (C) % suppression by iTregs in DMSO- or LLL12b-treated group from three MS patients (Teff:Treg=16:1) was summarized and compared with a paired Student's t test.

Acknowledgements

I would like to thank all the members of my Honors Thesis committee, especially my mentor Dr. Yuhong Yang, whose guidance and support helped me mature as a young scientist. I thank my lab mentors Saba Aqel and Emma Kraus for not only teaching me lab techniques but also believing in me. Finally, I thank my lab-mate Matthew Jeffers, who was always eager to talk with me about science or sports.

Our collaborator Chenglong Li (University of Florida) designed and synthesized LLL12b. Dr. Michael Racke and Dr. Amy Lovett-Racke provided the frozen human samples from MS patients. Yue Liu provided general lab support.

This work was supported by an NIH grant (1R01NS088437-01A1), two College of Arts and Sciences Undergraduate Research Scholarships and funding from the College of Arts and Sciences Undergraduate Research Apprentice Program.

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